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PRACTICAL APPLICATION OF ACETYLCHOLINE METHOD AS A NON-INJURIOUS MEANS TO OBTAIN EGGS FROM SEA URCHINS¹⁾

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In some marine biological laboratories where available sea urchins have become less abundant, it is earnestly advised to reduce the sacrifice of them in practical exercises of marine biology course for undergraduate students. Iida's method (1942) which has been usually employed to induce shedding of sea urchins, consists in replacing the perivisceral fluid with 0.5 M KCl after removal of the Aristotle's lantern, and the animals die inevitably after these treatments. In fact, no small number of sea urchins often have been sacrificed before a good shedding female is obtained. Recently Iwata and Fukase (1964 a and b) found that a very small amount of acetylcholine induced shedding in *Hemicentrotus pulcherrimus* and *Temnopleurus toreumaticus*; but shedding due to acetylcholine was not so vigorous, occasionally done from only some of gonopores and, without pretreatment with eserine, an inhibitor of acetylcholine esterase, shedding ceased within 1–3 minutes. Although these authors have carried out their studies with acetylcholine of concentration as low as 10^{-7} – 10^{-10} M, their results seem to suggest that vigorous and prolonged shedding could be induced when the amount of acetylcholine is large enough to keep its concentration above the threshold against the action of acetylcholine esterase in the perivisceral fluid or in the gonad. If so, and further provided that sea urchins could survive and repeat to shed regularly in a lapse of time after injection of such a large amount of acetylcholine, we would have another non-injurious method for inducing shedding in sea urchins.

The present experiments were designed to secure such a practical information and carried out during my summer course of Marine Zoology in 1972 at the Seto Marine Biological Laboratory. The work was suggested by Dr. Takasi Tokioka who has been deeply interested in the problems balancing supply and preservation of experimental animals. I am indebted also to Mr. Chuichi Araga for his courtesy in collecting and taking care of the experimental sea urchins.

Experiments and Results

Experiments were carried out on *Echinometra mathaei* and were partly repeated also

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on *Anthocidaris crassispina*, but the following results, unless otherwise described, were obtained on *Echinometra mathaei*.

For acetylcholine chloride (ACh in short hereafter, MW. 181. 67), 1 per cent solution corresponds to 5.6×10^{-2} M. Then, 1 ml of 0.5 per cent, 1 per cent and 2 per cent solution of ACh, i.e., 5 mg, 10 mg and 20 mg of ACh, were injected through the peristome into the perivisceral cavity of each of 15, 15 and 13 sea urchins, respectively.

In 34 seconds on the average (range 5–60 seconds) after an injection of 10 mg or 20 mg of ACh, fairly vigorous shedding was induced from all of 5 gonopores and continued for more than 15 minutes in most cases. But in the case when 5 mg of ACh was given, shedding was induced less vigorously and its duration was also shorter.

In 10–20 minutes after the injection the sea urchins became immobile, with tube feet hardly sticking to the substratum and with spines faintly responsive to external stimuli. The sea urchins recovered from such a locomotor disturbance in 5–15 hours, but they were reared for about 2 weeks in aquaria, being regularly fed with sea weeds to continue further observations.

On the 13th day after injection, the sea urchins still living were received the second injection of ACh to see whether they could shed again and whether they could still survive after the second injection.

The results obtained are shown in Table 1.

Table 1. Efficacy on shedding induction of ACh-injection and its effect on life in *Echinometra mathaei*.

	Dose mg of ACh	Number of sea urchins						
		received 1st injection	shedded by 1st injection	died in 12 days following 1st inject.	received 2nd injection	shedded by 2nd injection	died in 12 days following 2nd inject.	survived 12 sdays after 2nd inject.
Group A	5	15	14(7♂, 7♀)	7	8	7(3♂, 4♀)	0	8
Group B	10	15	15(9♂, 6♀)	1	14	14(9♂, 5♀)	0	14
Group C	20	13	13(5♂, 8♀)	2	11	9(4♂, 5♀)	0	11

Of 15 sea urchins received an injection of 5 mg of ACh, seven were found dead in 12 days after the first injection, thus the highest death rate was seen in the group of the smallest dose of ACh among three groups. However, this was probably due to an improper care of sea urchins before injection; as to avoid spontaneous shedding, they had been left exposed in a sink for several hours after collection getting wet by only sprinkling sea water. Experimental animals of the other two groups had been kept before injection in aquaria provided with running sea water, and then the death rate was as low as 7 per cent (1 out of 15) for the group B and 15 per cent (2 out of 13) for the group C (Table 1), respectively. Further, after the second injection, none was found dead in all three groups in the following days till the last day of the present

observation. These results represented that the sea urchins having received an injection of 5–20 mg of ACh, could live normally, when they were kept in natural or proper circumstances.

Shedding, similar to the first one, could be induced again by the second injection, if it was performed after a lapse of shedding for about 2 weeks; shedding did not resume when the injection was repeated immediately after prolonged shedding due to the preceding injection was over.

As the eggs obtained by the second injection developed normally to plutei as well as those obtained by the first injection, it is apparent that ACh would produce little effect on regular maturation of gametes and that the majority of sea urchins ever given an ACh injection would shed another batch of gametes matured after the previous injection.

In *Anthocidaris crassispina*, the second shedding was confirmed also after a lapse of about 2 weeks and the death rate was similarly low, if proper cares were taken to the animals before and after the first treatment.

Discussion and Conclusion

The electrical method developed by Iwata (1950) and by Harvey (1953) is an excellent method to obtain eggs without sacrificing sea urchins. However, this method seems to be employed in practice, only at the laboratories where necessary equipments are available.

The present ACh method which was also developed originally by Iwata and Fukase (1964 a), can be carried out with only an injection needle and a tuberculin syringe. And further, the method is very simple and would be actually effective to minimize the sacrifice of sea urchins for student exercises, if Tahara's sexing technique (1958) is applied beforehand. The present method, therefore, would be certainly recommendable at any laboratory where sea urchins are becoming scarce to be offered for the student exercise.

As a conclusive dose, 1 ml of 1 per cent solution of ACh is recommended for *Echinometra* and *Anthocidaris* and probably 1 ml of 0.5 per cent for such smaller species as *Mespilia globulus*.

Incidentally, it was found that the 1 per cent ACh solution in 5 per cent NaH_2PO_4 , sealed in ampullae and boiled for 5 minutes in a water bath, can be stored in the refrigerator with little loss of activity, because 5 per cent NaH_2PO_4 will keep a pH level at which ACh is stable and further 5 per cent NaH_2PO_4 gives little ill effect on sea urchins.

The injection may be performed with a tuberculin syringe into the perivisceral cavity through the peristome of any sea urchin which has been checked the sex beforehand and is placed the aboral side down in a flask filled up with sea water. Cutting spines has to be avoided, because this often results in a decrease in survival after the exhausted sea urchins are released again in the sea.

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